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Effect of cAMP on the activity and the phosphorylation of Na^+,K^+-ATPase in rat thick ascending limb of Henle

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Background. In rat kidney medullary thick ascending limb of Henle's loop (MTAL), activation of protein kinase A (PKA) was previously reported to inhibit Na^+,K^+-ATPase activity. This is paradoxical with the known stimulatory effect of cAMP on sodium reabsorption. Because this inhibition was mediated by phospholipase A_2 (PLA_2) activation, a pathway stimulated by hypoxia, we evaluated the influence of oxygen supply on cAMP action on Na^+,K^+-ATPase in MTAL.

Methods. Ouabain-sensitive ^{86}Rb uptake and Na^+,K^+-ATPase activity were measured in isolated MTALs. Cellular ATP content and the phosphorylation level of Na^+,K^+-ATPase were determined in suspensions of outer medullary tubules. Experiments were carried out under nonoxygenated or oxygenated conditions in the absence or presence of PKA activators.

Results. cAMP analogues or forskolin associated with 3-isobutyl-1-methylxanthine (IBMX) inhibited ouabain-sensitive ^{86}Rb uptake in nonoxygenated MTALs. In contrast, when oxygen supply was increased, cAMP stimulated ouabain-sensitive ^{86}Rb uptake and Na^+,K^+-ATPase activity. Improved oxygen supply was associated with increased intracellular ATP content. The phosphorylation level of the Na^+,K^+-ATPase α subunit was increased by cAMP analogues or forskolin associated with IBMX in oxygenated as well as in nonoxygenated tubules. Under nonoxygenated conditions, the inhibition of Na^+,K^+-ATPase was dissociated from its cAMP-dependent phosphorylation, whereas under oxygenated conditions, the stimulatory effect of cAMP analogues on ouabain-sensitive ^{86}Rb uptake was linearly related and cosaturated with the level of phosphorylation of the Na^+,K^+-ATPase α subunit.

Conclusion. In oxygenated MTALs, PKA-mediated stimulation of Na^+,K^+-ATPase likely participates in the cAMP-dependent stimulation of sodium reabsorption. Under nonoxygenated conditions, this stimulatory pathway is likely overridden by the PLA_2-mediated inhibitory pathway, a possible adaptation to protect the cells against hypoxic damage.

Active reabsorption of NaCl along the thick ascending limb (TAL) of mammalian kidneys is an essential process for salt and water homeostasis because it underlies the kidney's ability to either dilute or concentrate the urine. *In vitro* microperfusion studies [1, 2] have demonstrated that hormones coupled to the activation of the adenylyl cyclase/cyclic AMP (cAMP)/protein kinase A (PKA) cascade are the main effectors of the stimulation of NaCl reabsorption in the TAL. In TAL cells, as in all tubular cells, NaCl reabsorption is primarily energized by the basolateral Na^+,K^+-ATPase, which extrudes intracellular Na^+ ions into the peritubular compartment. Therefore, the previously reported inhibition of Na^+,K^+-ATPase activity by cAMP in microdissected MTALs [3, 4] appears paradoxical. Because we have previously shown that in rat proximal tubules, the activation of protein kinase C was associated with either inhibition or stimulation of Na^+,K^+-ATPase depending on oxygen availability [5], we hypothesized that the previously described inhibition of Na^+,K^+-ATPase by cAMP might be explained by cellular hypoxia. Therefore, the first aim of this study was to compare the effects of cAMP on Na^+,K^+-ATPase activity in MTALs under hypoxic and well-oxygenated conditions. Results confirmed the inhibitory effect of cAMP on MTAL Na^+,K^+-ATPase under hypoxic conditions and demonstrated a stimulation in well-oxygenated MTALs.

Previous studies on both purified Na^+,K^+-ATPase [6–9] and Na^+,K^+-ATPase expressed in COS-7 cells [10, 11] have shown that the catalytic α subunit of Na^+,K^+-ATPase can be phosphorylated by PKA. Thus, the second aim of this study was to determine whether cAMP induces a phosphorylation of Na^+,K^+-ATPase α subunit in medullary TALs (MTALs) and, if so, whether this process is related to the pump activity. Results indicate that in oxygenated as well as in nonoxygenated MTALs,

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PKA stimulation increased the phosphorylation level of the Na^+,K^+-ATPase α subunit. However, under hypoxic conditions, the inhibition of Na^+,K^+-ATPase was dissociated from its PKA-dependent phosphorylation, whereas under well-oxygenated conditions, the increase in Na^+,K^+-ATPase phosphorylation was correlated and cosaturated with the stimulation of its activity.

METHODS

Preparation of medullary thick ascending limbs

Studies were performed either on MTAL-enriched tubular suspensions or on microdissected MTAL from male Wistar rats (body wt 150 to 200 g). Animals were anesthetized with pentobarbital sodium (5 mg/100 g body wt, i.p.). For microdissection of single MTAL, the left kidney was perfused with 4 ml of incubation solution [120 mM NaCl, 5 mM RbCl, 4 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2 mM NaH_2PO_4, 0.15 mM Na_2HPO_4, 5 mM glucose, 10 mM lactate, 1 mM pyruvate, 4 mM essential and nonessential amino acids, 0.03 mM vitamins, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and 0.1% (wt/vol) bovine serum albumin (BSA), pH 7.45] supplemented with 0.18% (wt/vol) collagenase (CLS II, 0.87 U/mg; Serva, Vienna, Austria). The kidney was sliced into small pyramids that were subsequently incubated for 20 minutes at 30°C in an aerated incubation solution containing 0.05% (wt/vol) collagenase. After washing the pyramids, microdissection was performed at 0 to 4°C under stereomicroscopic control conditions.

The suspension of outer medullary tubules was prepared as previously described for renal cortex [12]. The kidneys were rapidly perfused with an ice-cold incubation solution. The inner stripe of outer medulla of the two kidneys was isolated and minced on ice to a pastelike consistency. Fragments of outer medullary tubules were obtained by placing the tissue on ice and pushing it by gentle pressure with a glass tube through graded sieves (150 and 100 μm in pore sizes). The tubule fragments were collected in 10 ml of ice-cold incubation solution, and after centrifugation, the pellet was resuspended in 5 ml of either oxygenated (95% O_2/5% CO_2) or nonoxygenated ice-cold incubation solution. Fragments of MTAL accounted for approximately 90% of the tissue mass in this preparation.

Well-oxygenated conditions were obtained by bubbling the incubation solution with 95% O_2/5% CO_2 just before dissection and incubation. This procedure does not significantly alter the pH of the incubation solution (pH without 95% O_2/5% CO_2, 7.45 ± 0.01; pH with 95% O_2/5% CO_2, 7.42 ± 0.02). In contrast, hypoxic conditions were obtained by using the incubation solution immediately (without oxygen bubbling), as this procedure was reported to induce lactate dehydrogenase release, a piece of evidence for cellular hypoxia [13].

$^{86}Rb^+$ uptake

The transport activity of Na^+,K^+-ATPase was estimated on isolated MTALs by measuring the ouabain-sensitive $^{86}Rb^+$ uptake under initial rate conditions in the presence of 5 mM Rb^+ as cold carrier, as previously described [14]. The osmolarity of incubation solution (discussed earlier in this article) was adjusted to 500 mOsm by the addition of mannitol to mimic the high osmotic pressure prevailing *in vivo* in the kidney medulla. This osmotic pressure was previously described to permit optimal measurement of $^{86}Rb^+$ uptake in MTALs [14]. Ten segments of MTAL were transferred within 1 μl of incubation solution into the concavity of a sunken bacteriological slide. After the addition of another 1 μl of incubation solution with or without drugs at twofold of their final concentration and/or 5 mM ouabain, tubules were preincubated at 37°C for various times. This preincubation period allowed the restoration of the transmembrane ion gradients, as well as the action of ouabain and drugs. $^{86}Rb^+$ uptake was determined after the addition of 0.5 μl incubation solution containing $^{86}Rb^+$ (Amersham, Little Chalfont, Buckinghamshire, UK) and was preequilibrated at 37°C. Incubation was stopped after 30 seconds by adding 30 μl of ice-cold rinsing solution (in mM: 150 choline chloride, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 2 $BaCl_2$, 5 HEPES, and mannitol up to 500 mOsm, pH 7.45). The tubules of each slide were then rapidly rinsed in three successive baths of ice-cold rinsing solution and were individually transferred with 0.2 μl of the last rinsing bath on a small microscope cover slip. After determination of its length by photography, each sample was dropped into a counting vial containing 0.5 ml of 1% (wt/vol) deoxycholic acid, and its radioactivity was measured by liquid scintillation. In each experiment, the blank value that was subtracted from all values was determined as the mean radioactivity of 8 to 10 replicate samples consisting of 0.2 μl of the last rinsing solution.

Ouabain-sensitive $^{86}Rb^+$ uptake was calculated as the difference between the mean values measured in samples without ouabain and with ouabain, respectively. $^{86}Rb^+$ uptake was expressed either as picomoles $Rb^+ \cdot mm^{-1} \cdot min^{-1}$ ± SE or as a percentage ± SE of the control (absence of PKA modulator).

Na^+,K^+-ATPase activity

The hydrolytic activity of Na^+,K^+-ATPase was determined in microdissected MTALs according to the previously described radiochemical assay [15] based on the measurement of Pi released from γ^{32}P-ATP. Briefly, each MTAL was individually transferred with 1 μl of incubation solution (discussed earlier in this article) into the concavity of a sunken bacteriological slide coated with dried BSA. The length of each tubule, which serves as reference for ATPase activity, was determined by photography. After the addition of another 1 μl of incubation

solution containing or not dibutyryl-cAMP at twice its final concentration (10^{-3} M), the samples were preincubated at 37°C for 15 minutes. The tubules were then thoroughly rinsed with 20 mM ice-cold tris(hydroxymethyl) aminomethane (Tris)-HCl (pH 7.4) and permeabilized by freezing/thawing in 0.2 μl of Tris-HCl. After the addition of 1 μl of ATPase assay solution (see composition later in this article), samples were incubated for 15 minutes at 37°C. Incubation was stopped by cooling and by the addition of 5 μl of 5% (wt/vol) cold trichloracetic acid. Samples were then transferred into 2 ml of 10% (wt/vol) activated charcoal. After mixing and centrifugation, the radioactivity was measured by liquid scintillation on 500 μl aliquots of supernatant, which contained the Pi formed from ATP.

The ATPase assay solution contained the following (in mM): 100 NaCl, 10 KCl, 10 $MgCl_2$, 1 ethylenediaminetetraacetic acid, 100 Tris-HCl, 10 MgATP, and tracer amounts (5 nCi/μl) of γ^{32}P-ATP (2 to 10 Ci/mmol; Dupont de Nemours, Boston, MA, USA) for total ATPase activity. For basal Mg^{2+}-ATPase activity measurements, NaCl was omitted, 1 mM ouabain was added, and the osmolarity was adjusted by the addition of choline-chloride. The pH of both solutions was 7.4.

In each experiment, total ATPase activity and Mg^{2+}-ATPase activity were each determined on five to seven replicates. Na^+,K^+-ATPase was taken after subtracting the mean Mg^{2+}-ATPase activity from the mean total ATPase activity and was expressed as pmol ATP · min^{-1} · mm^{-1} ± SE.

Adenosine triphosphate content

Suspensions of outer medullary tubules were incubated for 15 minutes at 37°C in the absence or presence of 10^{-3} M dibutyryl-cAMP (db-cAMP) under nonoxygenated or oxygenated incubation solution (discussed earlier in this article). After centrifugation and rapid aspiration of the incubation solution, the tubules were lyzed in 1 ml of ice-cold 0.4 N $HClO_4$. The samples were then centrifuged at 4°C. The supernatants were saved, and the pellets were solubilized in 100 μl of 0.8 N NaOH and 200 μl of 1% (wt/vol) Na-deoxycholate prior to determination of protein content by the bicinchoninic acid method with the bicinchoninic acid assay (Pierce, Rockford, IL, USA). After neutralization with 2 N K_2CO_3 for 30 minutes at 4°C, the supernatants were centrifuged once again at 4°C, and 50 μl aliquots were transferred into plastic vials containing 2 ml of the assay solution (100 mM Na_2HAsO_4, 20 mM $MgSO_4$, pH 7.4) and 50 μg/ml firefly luciferin-luciferase (Sigma, St. Louis, MO, USA). The emission of light was measured for 30 seconds in a luminometer (Lumat LB9507; Berthold, Wildbad, Germany). For each experiment, a standard curve was generated with MgATP (from 0 to $4 \cdot 10^{-6}$ M), and measurements were done in triplicate samples. Results were expressed as nmol ATP · mg $protein^{-1}$ ± SE.

Radiolabeling and incubation

Suspensions of outer medullary tubules were centrifuged for three minutes at 4°C, and the pellet was resuspended in 1.0 ml of incubation solution (discussed earlier in this article) containing 1 mCi/ml [^{32}P] orthophosphate (New England Nuclear, Danvers, MA, USA) and was incubated for two hours at 30°C. After three minutes of centrifugation at 4°C, the radioactive incubation solution was washed out, and the tubules were resuspended in the same medium without ^{32}Pi. The tubular suspension was then divided into 100 μl aliquots, and 100 μl of fresh incubation solution containing or not containing various agents was added. After incubation for various times at 37°C, the reaction was stopped by five minutes of centrifugation at 4°C. The pellet was then homogenized in 500 μl of ice-cold lysis buffer [20 mM Tris-HCl, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 30 mM NaF, 30 mM $Na_4O_7P_2$, 1 mM Na_3VO_4, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml leupeptin, 4 μg/ml aprotinin, and 1% Triton X-100, pH 7.45]. Protein content was determined by the bicinchoninic acid method with the BCA assay (Pierce).

Immunoprecipitation

Identical amounts of cellular protein (100 μg) were incubated overnight at 4°C with 10 μl of rabbit polyclonal anti–Na^+,K^+-ATPase antibody added to saturating amounts of protein A-sepharose beads (Pharmacia, Uppsala, Sweden), as described previously [12]. The immune complexes were centrifuged and washed four times with 1 ml of ice-cold lysis buffer, followed by resuspension in 100 μl of sample buffer [5% sodium dodecyl sulfate (SDS), 140 mM Tris, 2.5% β-mercaptoethanol, 6.8% sucrose, and 0.003% bromophenol blue]. Then the samples were heated for 15 minutes at 65°C.

Autoradiography

Proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 7% polyacrylamide gels using a running buffer containing 25 mM Tris-HCl, 192 mM glycine, 1% SDS, pH 8.75. Electrophoresis was performed at 350 V at 15°C, and proteins were then electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA, USA) at 100 V for three hours at 4°C in transfer buffer (25 mM Tris, 192 mM glycine, and 2% methanol). Membranes were dried and submitted to autoradiography with Hyperfilm-MP (Amersham) for two to six days at −70°C. Quantitation of autoradiograms was performed using a Molecular Dynamics laser-scanning densitometer and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Because this methodology does not allow the measurement of the stoichiometry of phos-

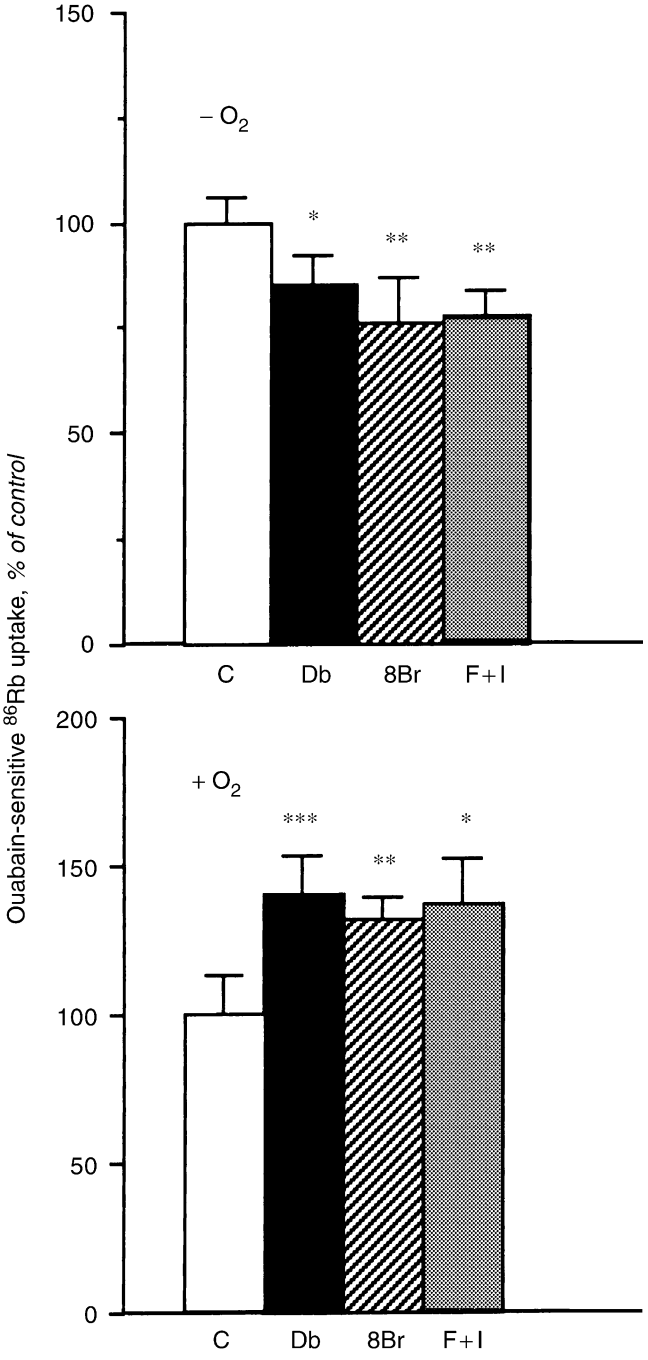


Fig. 1. Oxygen supply modulates the response of Na^+,K^+-ATPase to protein kinase A (PKA) activation. The initial rate of ouabain-sensitive $^{86}Rb^+$ uptake was measured in microdissected MTALs preincubated for 15 minutes at 37°C in the absence (C) or the presence of either 10^{-3} M db-cAMP (Db), 10^{-3} M 8br-cAMP (8Br), or 10^{-5} M forskolin and 10^{-4} M IBMX ($F + I$) under nonoxygenated ($-O_2$) or oxygenated ($+O_2$) conditions. Values are expressed as a percentage of controls and are means ± SE from 5 to 11 independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ vs. control.

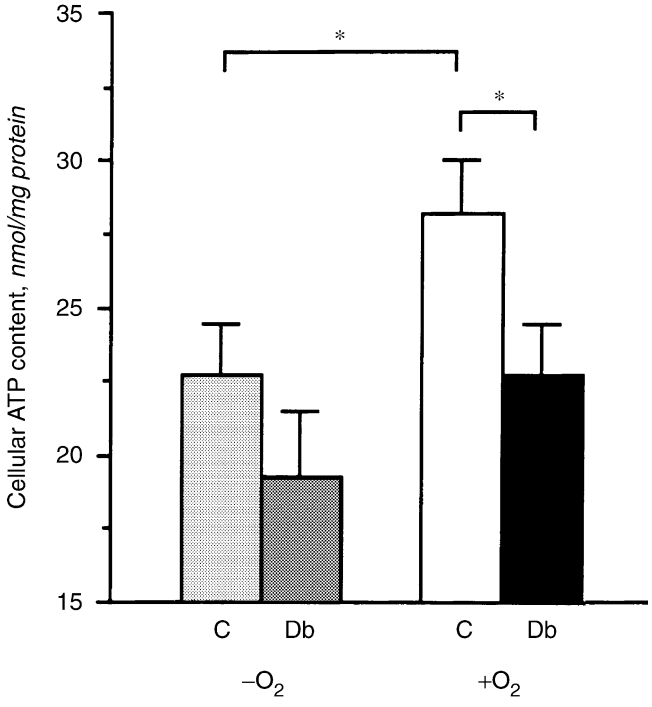


Fig. 2. Cellular adenosine 5′ triphosphate (ATP) content is increased by oxygen supply. Suspensions of outer medullary tubules were incubated for 15 minutes at 37°C in the absence (C) or presence of 10^{-3} M db-cAMP (Db) under nonoxygenated ($-O_2$) or oxygenated ($+O_2$) conditions before measurement of ATP content (**Methods** section). Values are expressed as nmol ATP · mg protein^{-1} and are means ± SE from 12 independent experiments. $^*P < 0.05$.

phorylation, results were expressed either as a percentage ± SE or as a fraction ± SE of the control optical density (absence of PKA modulator).

Immunoblotting

After rehydration, the PVDF membranes were blocked for one hour at room temperature in TBS-Tween (150 mM NaCl, 50 mM Tris, and 0.2% Tween 20, pH 7.5) supplemented with 3% BSA (wt/vol). After three washes in TBS-Tween, membranes were incubated for two hours at room temperature with a 1:200 (vol/vol) dilution of McK1 antibody, a mouse monoclonal antibody directed against Na^+,K^+-ATPase α_1 subunit [16]. The excess of antibody was removed by three washes in TBS-Tween, and membranes were then incubated with a second antimouse immunoglobulins antibody coupled to horseradish peroxidase (Amersham) at a dilution of 1:20,000 (vol/vol). After three washes in TBS-Tween, the immunoreactivity was detected by the chemiluminescence method, according to the manufacturer's instructions (Amersham).

Statistics

Statistical analysis of Rb^+ uptakes, Na,K-ATPase activities, and ATP contents were done by unpaired Student *t*-test or by analysis of variance for comparison of two or more than two groups, respectively. Statistical analysis of α subunit phosphorylation was done by the

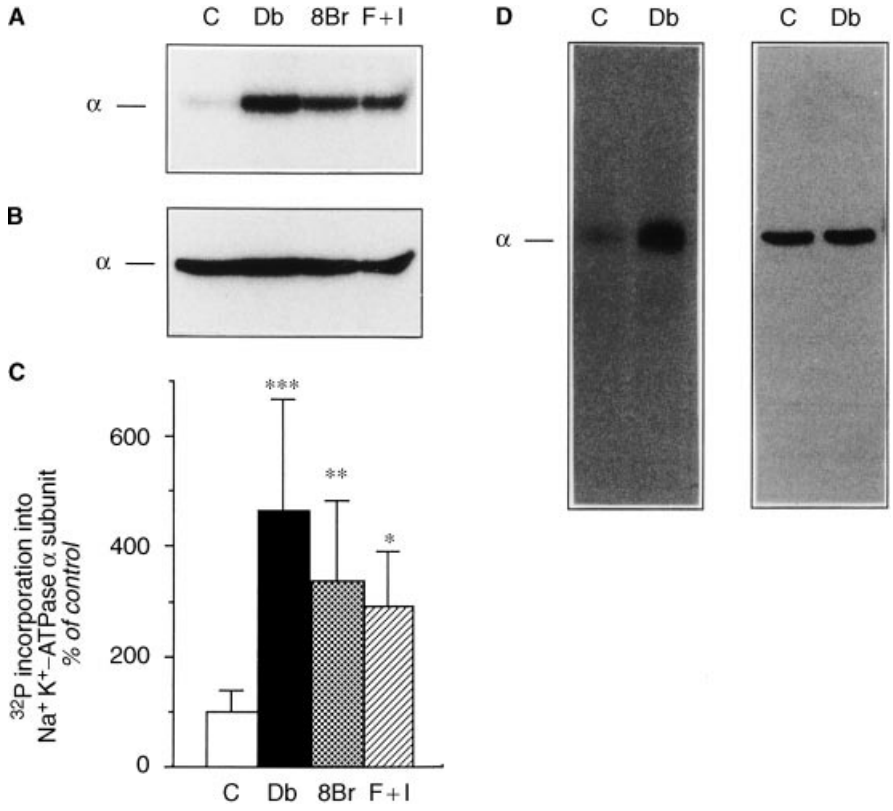


Fig. 3. PKA activation increases the phosphorylation level of Na^+,K^+-ATPase α subunit under oxygenated conditions. After metabolic [^{32}P]-labeling, suspensions of outer medullary tubules (*A*, *B*, and *C*) or isolated MTAL segments (*D*) were incubated for 15 minutes at 37°C in the absence (C) or presence of either 10^{-3} M db-cAMP (Db), 10^{-3} M 8br-cAMP (8Br), or 10^{-5} M forskolin and 10^{-4} M IBMX (*F* + I). (A) Autoradiogram showing the level of phosphorylation of Na^+,K^+-ATPase α subunit. (B) Immunoblot of the membrane shown in (A) with an antibody against Na^+,K^+-ATPase α_1 subunit (McK1) showing that similar amounts of Na^+,K^+-ATPase were present in each lane. (C) Densitometric quantitation of ^{32}P incorporation into Na^+,K^+-ATPase α subunit. Values are expressed as a percentage of control and are means ± SE from seven independent experiments such as that shown in (A). $^*P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.005$ vs. control. (D) Autoradiogram (left panel) and immunoblot (right panel) of the Na^+,K^+-ATPase α subunit from ^{32}P-labeled isolated MTALs.

Mann–Whitney *U*-test or by the Kruskal–Wallis test for comparison of two or more than two groups, respectively. Results are expressed as means ± SE from *N* independent experiments. Each experiment was performed with tubules from one animal. *P* values less than 0.05 were considered significant.

RESULTS

Influence of oxygen supply on the control of Na^+,K^+-ATPase by protein kinase A activators

In this first series of experiments, the effect of the activation of PKA on Na^+,K^+-ATPase was compared in MTALs under oxygenated and nonoxygenated conditions. The PKA pathway was stimulated by preincubating microdissected MTALs for 15 minutes at 37°C in the presence of either cAMP analogues [10^{-3} M db-cAMP or 10^{-3} M 8-bromo-cAMP (8br-cAMP)] or forskolin (10^{-5} M) plus 3-isobutyl-1-methylxanthine (IBMX, 10^{-4} M). Results in Figure 1 confirm that under nonoxygenated conditions (Fig. 1A), activation of PKA inhibited ouabain-sensitive ^{86}Rb uptake by 15 to 25% (as pmol · mm^{-1} · min^{-1} ± SE: control, 25.7 ± 1.5 [11]; db-cAMP, 20.6 ± 1.6 [9], $P < 0.05$; 8br-cAMP: 17.7 ± 3.5 [5], $P < 0.01$; forskolin + IBMX: 18.3 ± 1.5 [6], $P < 0.01$). In contrast, under oxygenated conditions (Fig. 1B), the activation of PKA increased ouabain-sensitive ^{86}Rb uptake by 30 to 40% (as pmol · mm^{-1} · min^{-1} ± SE: control, 23.6 ± 3.0 [11]; db-cAMP, 34.0 ± 4.4 [9], $P < 0.001$; 8br-cAMP: 34.1 ± 5.5 [8], $P < 0.01$; forskolin + IBMX: 31.3 ± 3.2 [10], $P < 0.05$). Under both oxygenation conditions, ouabain-insensitive ^{86}Rb uptake was not altered by PKA activation (not shown).

Under nonoxygenated conditions, PKA activators primarily inhibited Na^+,K^+-ATPase, as evidenced by a decrease in maximal hydrolytic activity of the enzyme in permeabilized MTALs [3, 4]. Therefore, the following experiments were aimed at characterizing the mechanism of Na^+,K^+-ATPase stimulation by cAMP under well-oxygenated conditions. Because ouabain-sensitive ^{86}Rb uptake was measured in intact cells, its stimulation in response to PKA activation could be theoretically achieved through either a primary effect on Na^+,K^+-ATPase or an increase in intracellular Na^+ concentration secondary to a stimulation of Na^+ entry. To discriminate between these two possibilities, the hydrolytic activity of Na^+,K^+-ATPase was measured in permeabilized MTALs in the presence of saturating Na^+ concentration (100 mM). Within 15 minutes of incubation at 37°C, 10^{-3} M db-cAMP stimulated Na^+,K^+-ATPase activity by 35% (as pmol · mm^{-1} · min^{-1} ± SE: control, 33.4 ± 3.3; db-cAMP, 45.1 ± 2.9, $P < 0.05$; $N = 6$). Mg^{2+}-ATPase activity was not altered by db-cAMP (data not shown). This indicates that under well-oxygenated conditions,

activation of PKA stimulates Na^+,K^+-ATPase independently of Na^+ availability.

Effect of oxygen supply on cellular adenosine triphosphate content

Because the contribution of anaerobic metabolism is very low in MTAL cells [17], we explored whether the opposite effects of cAMP on Na^+,K^+-ATPase activity observed under nonoxygenated or well-oxygenated conditions were associated with differences in the cellular ATP content. As depicted in Figure 2, after 15 minutes of incubation at 37°C in the absence of PKA activator, oxygenation of the incubation solution increased cellular ATP content by 25% (as nmol ATP · mg protein^{-1} ± SE; $-O_2$, 22.7 ± 1.8; $+O_2$, 28.2 ± 1.8; $P < 0.05$; $N = 12$). These results are in good agreement with previous measurements performed in isolated MTALs [17, 18]. After 15 minutes at 37°C in the presence of 1 mM db-cAMP, the cellular ATP content measured under nonoxygenated and well-oxygenated conditions was decreased by 15 and 20% ($P < 0.05$), respectively. Assuming that (*a*) one molecule of ATP is hydrolyzed for two Rb ions transported and (*b*) one millimeter of MTAL contains approximately 50 ng protein (unpublished observations) [17, 18], the turnover rate of intracellular ATP due to Na^+,K^+-ATPase can be calculated from these ATP contents and from the ouabain-sensitive ^{86}Rb uptake given in Figure 1. In well-oxygenated MTAL cells, amounts of ATP equivalent to the total cellular pool were burned by Na^+,K^+-ATPase every seven and four seconds under basal and db-cAMP–stimulated conditions, respectively. In contrast, under nonoxygenated conditions, the Na^+,K^+-ATPase–dependent cellular ATP turnover was similar under control and db-cAMP–stimulated conditions (every 5.3 vs. 5.6 seconds).

Effect of protein kinase A activation on the phosphorylation level of Na^+,K^+-ATPase

In the following experiments, the effect of PKA activators on the phosphorylation level of the Na^+,K^+-ATPase α subunit was compared under oxygenated and nonoxygenated conditions. Under well-oxygenated conditions, after 15 minutes of incubation at 37°C in the presence of cAMP analogues or forskolin plus IBMX, the phosphorylation level of Na^+,K^+-ATPase α subunit immunoprecipitated from radiolabeled outer medullary tubules increased 2.9- to 4.6-fold (as a percentage of control ± SE: db-cAMP, 464 ± 204, $P < 0.005$; 8br-cAMP, 448 ± 149, $P < 0.01$; forskolin + IBMX, 292 ± 99, $P < 0.05$; $N = 7$; Fig. 3 A, C), whereas the amount of α_1 subunit detected by immunoblotting with McK1 was not changed (Fig. 3B). It is worth noting that a low basal level of phosphorylation of Na^+,K^+-ATPase α subunit was observed even in the absence of experimental activation of PKA. As depicted in Figure 3D, 10^{-3} M db-cAMP also

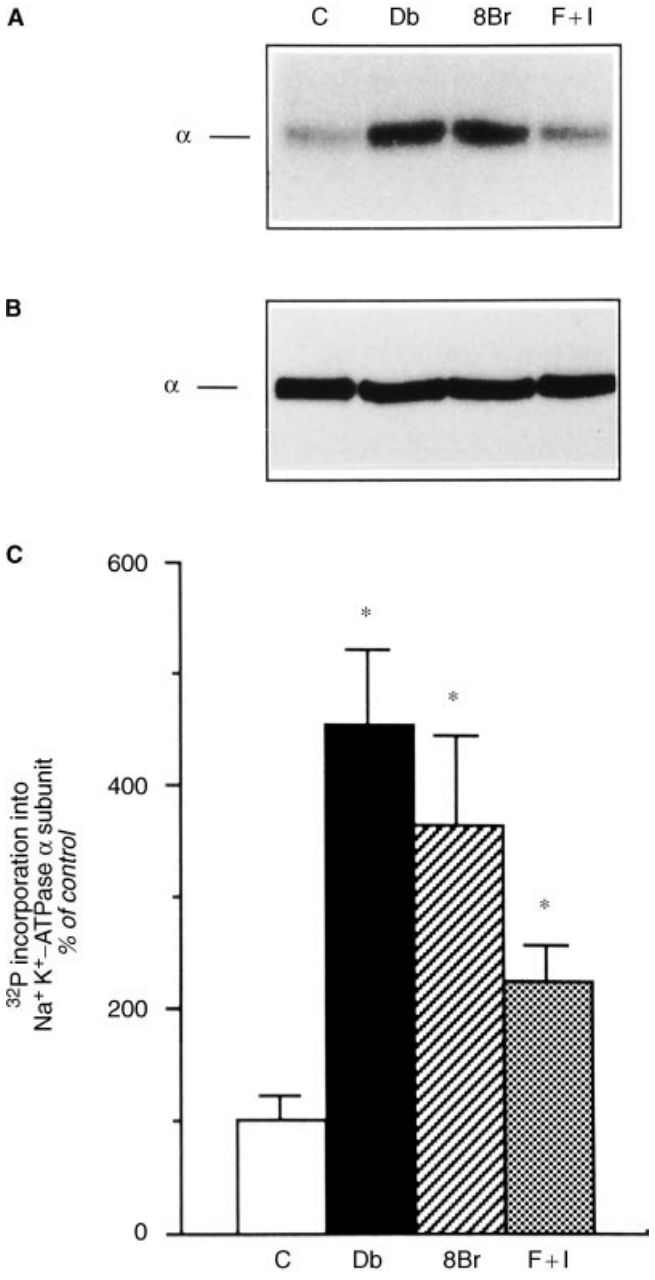
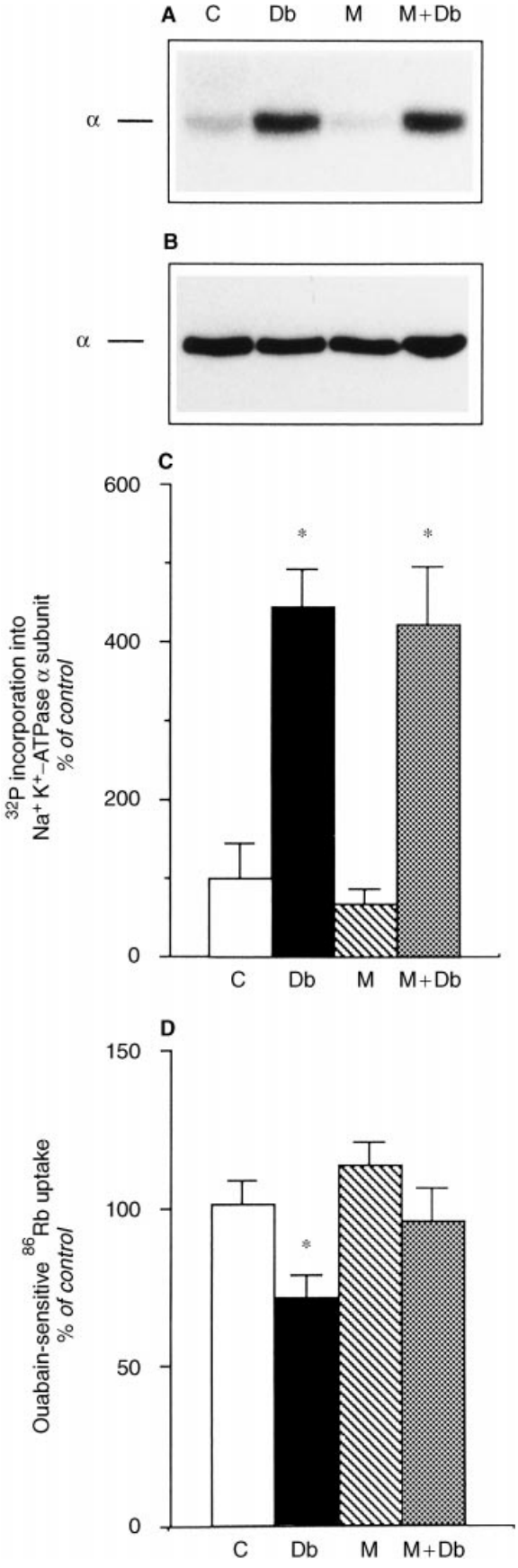


Fig. 4. Protein kinase A (PKA) activation increases the phosphorylation level of Na^+,K^+-ATPase α subunit under nonoxygenated conditions. After metabolic [^{32}P]-labeling, suspensions of outer medullary tubules (*A*, *B*, and *C*) were incubated for 15 minutes at 37°C in the absence (C) or presence of either 10^{-3} M db-cAMP (Db), 10^{-3} M 8br-cAMP (8Br), or 10^{-5} M forskolin and 10^{-4} M IBMX (*F* + I). (A) Autoradiogram showing the level of phosphorylation of Na^+,K^+-ATPase α subunit. (B) Immunoblot of the membrane shown in (A) with an antibody against Na^+,K^+-ATPase α_1 subunit (McK1) showing that similar amounts of Na^+,K^+-ATPase were present in each lane. (C) Densitometric quantitation of ^{32}P incorporation into Na^+,K^+-ATPase α subunit. Values are expressed as a percentage of control and are means ± SE from five independent experiments such as that shown in (A). *$P < 0.05$ vs. control.



increased the phosphorylation level of Na^+,K^+-ATPase α subunit approximately fourfold in MTALs microdissected from collagenase-treated kidneys. Because identical results were obtained using either microdissected MTALs or outer medullary tubule suspensions, this latter preparation was used in all subsequent immunoprecipitation experiments.

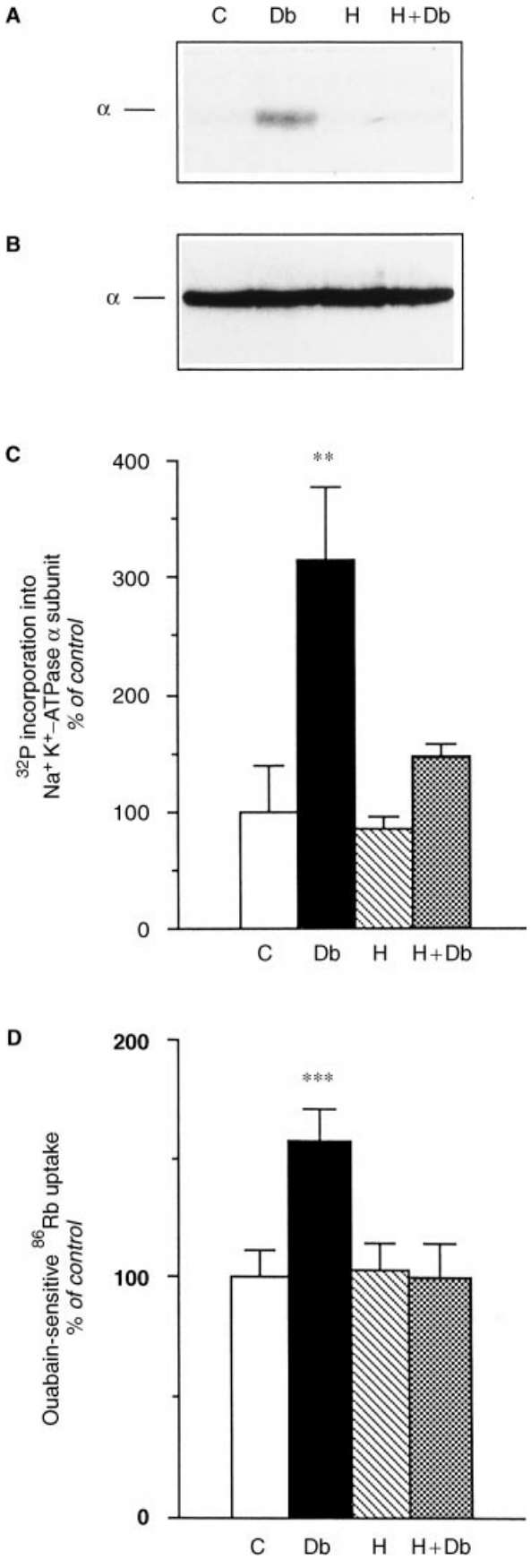
Under nonoxygenated conditions, the phosphorylation level of the Na^+,K^+-ATPase α subunit from outer medullary tubules was increased to the same extent as under well-oxygenated conditions (as a percentage of control ± SE: db-cAMP, 454 ± 69, $P < 0.01$; 8br-cAMP, 365 ± 80, $P < 0.01$; forskolin + IBMX, 237 ± 30, $P < 0.01$; $N = 5$; Fig. 4 A, C). As depicted by Figure 4B, the amount of α_1 subunit detected by immunoblotting with McK1 was similar in all experimental conditions.

The results demonstrate that activation of PKA increases the phosphorylation level of the Na^+,K^+-ATPase α subunit equally well in oxygenated and nonoxygenated MTAL.

Effect of mepacrine on the cAMP-induced inhibition of Na^+,K^+-ATPase under nonoxygenated conditions

Previous studies have suggested that cAMP-induced inhibition of Na^+,K^+-ATPase activity observed in nonoxygenated MTAL involves phospholipase A_2 (PLA_2)-dependent arachidonic acid generation and its subsequent metabolism into active compounds through the mono-oxygenase pathway [3, 4]. In these reports, the inhibitory effect of cAMP was fully prevented by mepacrine. Although mepacrine is not a highly specific PLA_2 inhibitor, this observation was taken as an indication of the role of PLA_2 in mediating the effect of cAMP. In the following experiments, mepacrine was used as a tool to determine whether the reversal of the inhibitory effect of cAMP on Na^+,K^+-ATPase was related to the modulation of the phosphorylation level of its α subunit. For

Fig. 5. Under nonoxygenated conditions, mepacrine does not interfere with the effect of db-cAMP on Na^+,K^+-ATPase phosphorylation but abolishes the inhibition of its transport activity. After metabolic [^{32}P]-labeling, identical amounts of outer medullary tubular suspension were preincubated for 30 minutes at 37°C either in control conditions (C) or in the presence of 10^{-3} M db-cAMP (Db), 10^{-5} M mepacrine (M), or mepacrine + db-cAMP (M + Db). (*A*) Autoradiogram showing that db-cAMP–induced increase in phosphorylation of Na^+,K^+-ATPase α subunit was not altered by mepacrine. (*B*) Immunoblot of the membrane shown in (A) with an antibody against Na^+,K^+-ATPase α_1 subunit (McK1) showing that similar amounts of Na^+,K^+-ATPase were present in each lane. (*C*) Densitometric quantitation of ^{32}P incorporation into Na^+,K^+-ATPase α subunit. Values are expressed as a percentage of control and are means ± SE from four independent experiments. $^*P < 0.05$ vs. control. (*D*) Ouabain-sensitive ^{86}Rb uptake in isolated MTALs preincubated under the same conditions discussed earlier here. Values are expressed as a percentage of control and are means ± SE from five independent experiments. $^*P < 0.05$ vs. control.



this purpose, we studied the effect of db-cAMP on both the phosphorylation and the activity of Na^+,K^+-ATPase in the presence or absence of mepacrine. As depicted in Figure 5 A and C, within 15 minutes after incubation at 37°C, 10^{-5} M mepacrine did not alter basal and db-cAMP–induced phosphorylation of Na^+,K^+-ATPase α subunit (as a percentage of control ± SE: db-cAMP, 451 ± 48, $P < 0.05$; mepacrine, 67 ± 20, NS; mepacrine + db-cAMP, 427 ± 76, $P < 0.05$; $N = 4$). The amount of α_1 subunit detected by immunoblotting was similar under these conditions (Fig. 2B). These results indicate that mepacrine-sensitive process is not involved in the PKA-induced phosphorylation of Na^+,K^+-ATPase.

The efficacy of mepacrine was checked by measuring its effect on the transport activity of Na^+,K^+-ATPase under the same experimental conditions. Figure 6D shows that incubation of microdissected MTALs with 10^{-5} M mepacrine completely abolished the inhibitory effect of 10^{-3} M db-cAMP on ouabain-sensitive ^{86}Rb uptake (as a percentage of control ± SE: db-cAMP, 71 ± 7, $P < 0.01$; mepacrine, 113 ± 7, $P < 0.05$; mepacrine + db-cAMP, 95 ± 11, NS; $N = 5$), confirming previous observations [3, 4]. It should be mentioned that in these experiments, mepacrine alone slightly but consistently stimulated Na^+,K^+-ATPase.

In summary, mepacrine prevented the inhibitory effect of cAMP on Na^+,K^+-ATPase activity but did not alter PKA-induced phosphorylation of the Na^+,K^+-ATPase α subunit. Therefore, in nonoxygenated MTALs, the effects of cAMP on phosphorylation and activity of Na^+,K^+-ATPase can be dissociated.

Effects of protein kinase A inhibitors on the phosphorylation level and activity of Na^+,K^+-ATPase

The role of PKA in mediating the effects of cAMP analogues on Na^+,K^+-ATPase was investigated by using H89, a specific inhibitor of PKA. As depicted in Figure

Fig. 6. Inhibition of protein kinase A curtails the stimulatory effects of db-cAMP on the phosphorylation level and the transport activity of Na^+,K^+-ATPase under oxygenated conditions. After metabolic [^{32}P]-labeling, identical amounts of outer medullary tubular suspension were preincubated for 30 minutes at 37°C either in control conditions (C) or in the presence of either 10^{-3} M db-cAMP (Db), 10^{-6} M H89 (H), or H89 + db-cAMP (H + Db). (*A*) Autoradiogram showing that db-cAMP–induced increase in phosphorylation of Na^+,K^+-ATPase α subunit was prevented by H89. (*B*) Immunoblot of the membrane shown in (A) with an antibody against Na^+,K^+-ATPase α_1 subunit (McK1) showing that similar amounts of Na^+,K^+-ATPase were present in each lane. (*C*) Densitometric quantitation of ^{32}P incorporation into Na^+,K^+-ATPase α subunit. Values are expressed as a percentage of control and are means ± SE from five independent experiments. $^{**}P < 0.01$ vs. control. (*D*) Ouabain-sensitive ^{86}Rb uptake in isolated MTALs preincubated under the same conditions as discussed earlier here. Values are expressed as a percentage of control and are means ± SE from seven independent experiments. $^{***}P < 0.005$ vs. control.

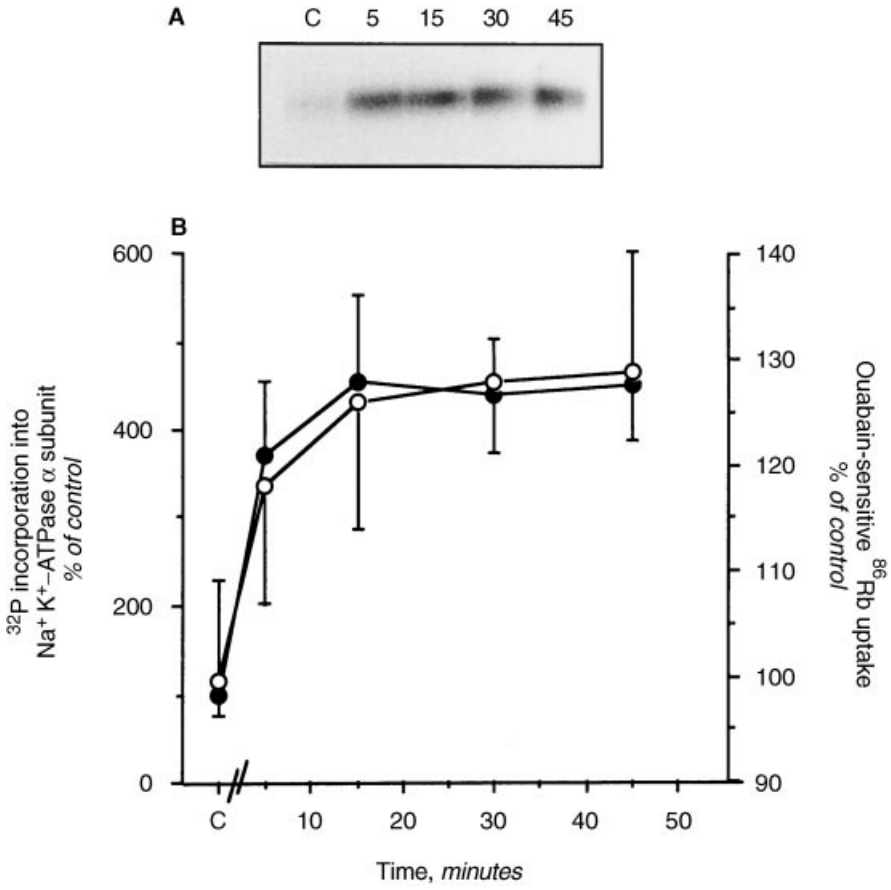


Fig. 7. Time course of db-cAMP action on the phosphorylation level and the transport activity of Na^+,K^+-ATPase under oxygenated conditions. (*A*) After metabolic [^{32}P] labeling, identical amounts of outer medullary tubular suspension were preincubated at 37°C for either 30 minutes in control conditions (C) or 5 to 45 minutes in the presence of 10^{-3} M db-cAMP. The autoradiogram shows that db-cAMP–induced a time-dependent increase in phosphorylation of Na^+,K^+-ATPase α subunit. (*B*) The phosphorylation level of the α subunit of Na^+,K^+-ATPase from outer medullary tubular suspension (●) and the ouabain-sensitive ^{86}Rb uptake by isolated MTALs (○) were determined after preincubation for various times in the presence of 10^{-3} M db-cAMP. Results are expressed as percentage of controls (C = preincubation for 45 minutes in the absence of db-cAMP) and are means ± SE from three to seven independent experiments.

6 A and C, preincubation of the tubules for 30 minutes in the presence of $5 \cdot 10^{-5}$ M H89 did not alter the basal phosphorylation of Na^+,K^+-ATPase α subunit but significantly reduced its db-cAMP–induced phosphorylation (as a percentage of control ± SE: db-cAMP, 314 ± 66, $P < 0.01$; H89, 85 ± 11, NS; H89 + db-cAMP, 147 ± 11, NS; $N = 5$). Similarly, Figure 6D shows that under well-oxygenated conditions, the stimulation of ouabain-sensitive ^{86}Rb uptake by 10^{-3} M db-cAMP was also blunted by H89 (in pmol · mm^{-1} · min^{-1} ± SE: control, 20.1 ± 2.4; db-cAMP, 28.4 ± 5.3, $P < 0.005$; H89, 21.4 ± 3.6, NS; H89 + db-cAMP, 20.5 ± 3.2, NS; $N = 7$). These data indicate that the effect of db-cAMP is mediated by PKA activation, whereas PKA does not account for the basal phosphorylation of the α subunit. H89 did not change the amount of the Na^+,K^+-ATPase α subunit (Fig. 6B) and the ouabain-insensitive ^{86}Rb uptake (not shown).

Time and concentration dependence of db-cAMP action on the Na^+,K^+-ATPase phosphorylation and ^{86}Rb uptake

To investigate whether under well-oxygenated conditions there might be a relationship between the stimulatory effects of PKA activation on the phosphorylation level of Na^+,K^+-ATPase on the one hand, and its activity on the other, we compared the time course and the concentration dependence of db-cAMP action on these two parameters. Figure 7 shows that within five minutes, 10^{-3} M db-cAMP increased both Na^+,K^+-ATPase phosphorylation and ouabain-sensitive ^{86}Rb uptake and that the stimulation of both parameters plateaued after 15 minutes of incubation with db-cAMP. The concentration dependence of the effects of db-cAMP is presented in Figure 8; 10^{-5} M db-cAMP was the threshold for stimulating both Na^+,K^+-ATPase phosphorylation and ouabain-sensitive ^{86}Rb influx, and further stimulation was observed up to 10^{-2} M db-cAMP.

To further assess whether there is a possible quantitative relationship between the effects of cAMP on the activity of Na^+,K^+-ATPase and its PKA-dependent phosphorylation, the fractional changes in ouabain-sensitive ^{86}Rb uptake (calculated as fractions of the maximal stimulation) were plotted as a function of the fractional changes in phosphorylation level of the α subunit. Figure 9, drawn from the data presented in Figures 7 and 8, shows that the level of PKA-dependent phosphorylation of Na^+,K^+-ATPase was linearly correlated ($r^2 = 0.97$) and was cosaturated with the stimulatory action of cAMP

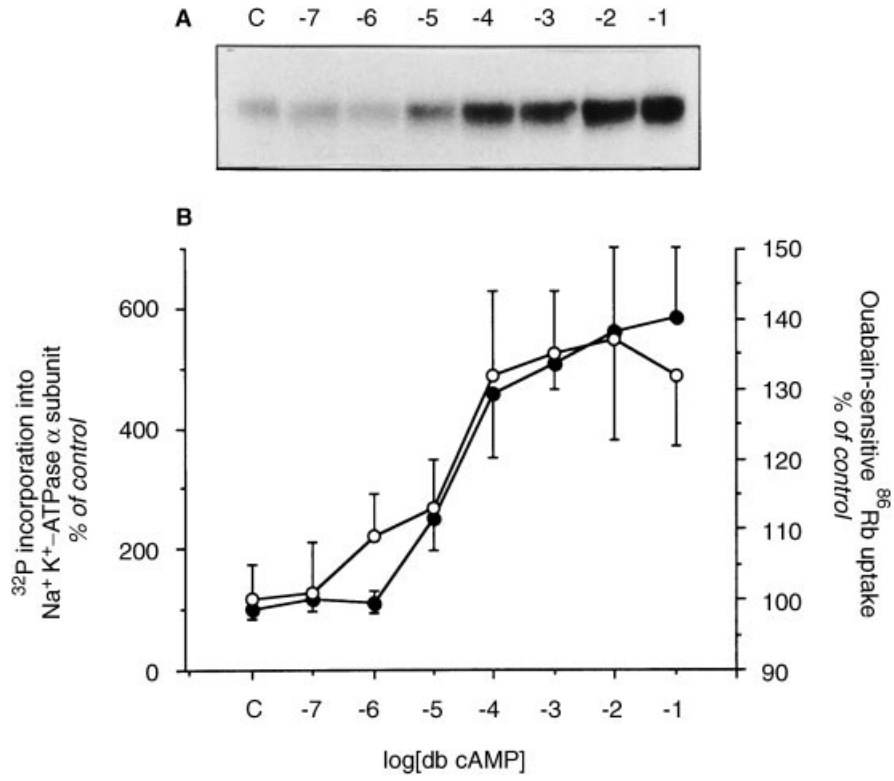


Fig. 8. Dose dependence of db-cAMP action on the phosphorylation level and the transport activity of Na^+,K^+-ATPase. (*A*) After metabolic [^{32}P]-labeling, identical amounts of outer medullary tubular suspension were preincubated for 30 minutes at 37°C either in control conditions (C) or in the presence of 10^{-7} to 10^{-1} M db-cAMP. The autoradiogram shows that db-cAMP induced a dose-dependent increase in phosphorylation of Na^+,K^+-ATPase α subunit. (*B*) The phosphorylation level of the α subunit of Na^+,K^+-ATPase from outer medullary tubular suspension (●) and the ouabain-sensitive ^{86}Rb uptake by isolated MTALs (○) was determined after preincubation in the presence of 10^{-7} to 10^{-1} M db-cAMP. Results are expressed as percentage of controls (C = preincubation in the absence of db-cAMP) and are the means ± SE from three to seven independent experiments.

on the pump activity. This analysis suggests that in MTALs, the stimulation of Na^+,K^+-ATPase on PKA activation might be related to the increase in the phosphorylation of its α subunit.

DISCUSSION

The results of this study bring new light on two distinct areas: (*a*) the role of oxygen availability on the regulation of MTAL by the PKA cascade and (*b*) the relationship between PKA-stimulated phosphorylation and Na^+,K^+-ATPase activity in intact cells.

Influence of oxygen supply

This work demonstrates that PKA activation inhibited Na^+,K^+-ATPase under normal *in vitro* conditions (absence of additional oxygenation), confirming previous findings [3, 4], whereas this activity was stimulated in well-oxygenated MTALs (Fig. 1). In these experiments, only the oxygenation status differed between the two conditions studied, whereas pH, extracellular calcium, and osmolarity were identical.

In MTAL cells, cellular ATP is almost exclusively supplied by oxidative metabolism, and the contribution of anaerobic metabolism is very low [17]. Therefore, the generation of ATP through metabolism of lactate and pyruvate provided by the incubation solution (**Methods** section) is dependent on oxygen availability. Oxygen supply is potentially an important rate-limiting factor for active Na^+ transport by MTAL because (*a*) Na^+,K^+-ATPase is responsible for as much as 80% of the oxygen consumption [19], and (*b*) measurements with microelec-

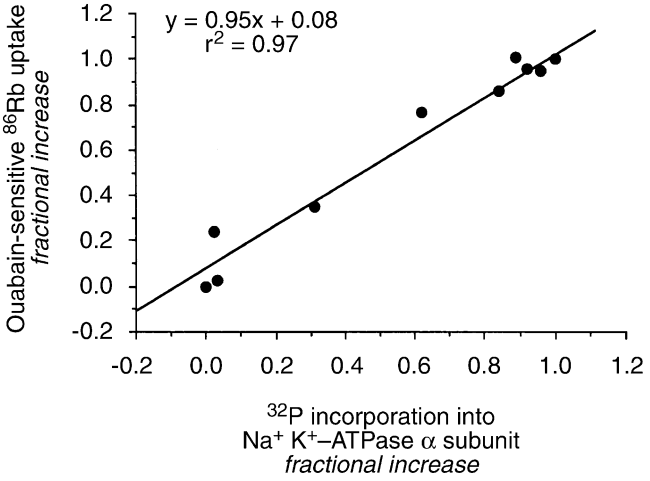


Fig. 9. Stimulation of the activity of Na^+,K^+-ATPase is linearly correlated and cosaturates with the phosphorylation level of its α subunit. The fractional increases in ouabain-sensitive ^{86}Rb uptake determined in single MTALs were plotted as a function of the fractional increases in ^{32}P incorporation in the α subunit of Na^+,K^+-ATPase from outer medullary tubular suspensions preincubated under the same conditions. Values were calculated from the experiments depicted in Figures 7 and 8; $Y = 0.95\times + 0.08$; $r^2 = 0.97$.

trodes implanted in the kidney parenchyma indicate that the low PO_2 prevailing in the kidney medullary region [20, 21] is mainly determined by the active reabsorption of NaCl, as inhibition of this transport by loop diuretics markedly increases PO_2 [22]. The dependence of Na^+,K^+-ATPase activity toward ATP synthesis through oxidative metabolism is further exemplified by the dose-dependent decrease in ouabain-sensitive ^{86}Rb uptake observed in MTALs exposed to the mitochondrial uncoupler carbonyl cyanide trifluoromethoxyphenylhydrazone [14]. This study shows that basal cellular ATP content is 25% lower in nonoxygenated tubules (Fig. 2), indicating that, at least *in vitro*, oxygen availability is rate limiting for ATP synthesis in MTAL. Because the ouabain-sensitive ^{86}Rb uptake measured under unstimulated conditions was not altered by the oxygenation status (Fig. 1), MTAL cells could face the energy demand to meet the basal active cation transport at the cost of an increased ATP turnover under the standard nonoxygenated conditions (discussed in the **Results** section).

The decrease in steady-state ATP level induced by cAMP under well-oxygenated conditions (Fig. 2) most likely reflects an increased ATP consumption by the Na^+,K^+-ATPase. This interpretation is strongly supported by the observed decrease in cellular ATP content induced by the stimulation of Na^+,K^+-ATPase by Na^+ ionophores in isolated MTALs [17, 18]. However, despite a decrease in cellular ATP content, stimulation of Na^+,K^+-ATPase was sustained, indicating that oxygen and metabolic supply was sufficient to meet the increase in cellular ATP turnover. In contrast, when the oxygen supply was not increased, cAMP inhibited Na^+,K^+-ATPase (Fig. 1) and further decreased the cellular ATP content (Fig. 2). The simplest explanation for these observations would be that a rate-limiting effect of cellular ATP concentration on Na^+,K^+-ATPase activity exists. However, this hypothesis can be ruled out because the hydrolytic activity of Na^+,K^+-ATPase determined in the presence of saturating concentrations of exogenous ATP is also decreased by cAMP under nonoxygenated conditions [3, 4]. Alternatively, the absence of cAMP-induced alteration in ATP turnover under nonoxygenated conditions could indicate that cellular hypoxia triggers some protective mechanisms leading to the inhibition of Na^+,K^+-ATPase and thereby prevents deep cellular ATP depletion. The inhibition of Na^+,K^+-ATPase activity observed under nonoxygenated conditions was previously shown to result from the stimulation of a PLA_2/cytochrome P450-dependent monoxygenase pathway and synthesis of arachidonic acid derivatives that directly inhibit the pump activity [3, 4]. Because this pathway may be activated by tubular hypoxia [13, 23], we propose that this regulatory pathway is not triggered directly by PKA stimulation, but rather results from decreased partial pressure of oxygen (PO_2) and subsequent inadequation of cellular ATP supply brought about by an initial increase in NaCl transport. Thus, whatever the oxygenation status of MTALs, activation of PKA would first stimulate NaCl reabsorption, a process that increases oxygen and ATP consumption, as indicated by the decrease in cellular ATP content (Fig. 2). In the absence of an adequate oxygen supply, stimulation of NaCl transport would rapidly provoke cellular hypoxia and insufficient ATP synthesis, which, in turn, would stimulate the PLA_2/arachidonate/mono-oxygenase inhibitory pathway. Conversely, when the oxygen supply is sufficient, the cell metabolism could face the additional demand for ATP synthesis elicited by a cAMP-induced increase in NaCl transport, and the PLA_2-arachidonate-mono-oxygenase pathway would not be triggered.

In fact, inhibition of Na^+,K^+-ATPase activity observed under nonoxygenated conditions should not be considered as paradoxical, but should be viewed as a defensive mechanism preventing the deleterious effects of anoxia and allowing cell survival during extreme conditions. This hypothesis is supported by previous results indicating that inhibition of active sodium transport reduces hypoxic injury [24] and that arachidonic acid protects kidney cells against anoxic death [25]. It is worth noting that this PLA_2-mediated inhibitory pathway reduces NaCl transport by inhibiting both the apical Na^+-K^+-2 Cl^- cotransporter [26] and basolateral Na^+,K^+-ATPase. This concerted regulation of the two transport systems allows maintenance intracellular Na^+ homeostasis.

The stimulation of Na^+,K^+-ATPase–mediated Rb^+ uptake observed in well-oxygenated MTALs is physiologically relevant because (*a*) cAMP-generating hormones increase NaCl reabsorption in MTAL [1, 27] and (*b*) the stimulation of Na,K-ATPase observed in response to cAMP (30 to 40% increase) is quantitatively similar to the vasopressin-induced increase in sodium reabsorption (38% increase) determined by *in vitro* microperfusion [28].

Previous studies have shown that both the apical Na^+-K^+-2 Cl^- cotransporter [29, 30] and the basolateral Cl^- channels [31] are molecular targets of the stimulatory effect of cAMP on NaCl reabsorption in rat MTALs. In fact, cAMP-induced stimulation of apical Na^+-K^+-2 Cl^- cotransporter is sufficient to account for the stimulation of NaCl reabsorption. Indeed, increasing apical Na^+ entry would raise intracellular Na^+ concentration ($[Na^+_i]$), which, in turn, would activate basolateral Na^+,K^+-ATPase, because $[Na^+_i]$ is normally rate limiting [32]. However, this finding that cAMP increased not only ouabain-sensitive Rb^+ uptake but also the V_{max} of Na^+,K^+-ATPase indicates that the pump itself was stimulated through activation of PKA, independently of changes in $[Na^+_i]$. The simultaneous stimulation of apical Na^+,K^+-2 Cl^- cotransport and basolateral Na^+,K^+-ATPase by cAMP permits an increase the transcellular flux of Na^+ without altering $[Na^+_i]$.

Phosphorylation of Na^+,K^+-ATPase

Over the past years, several studies using purified Na^+,K^+-ATPase preparations [6–8] and transfected cells [10, 11] indicated that the α subunit of Na^+,K^+-ATPase is phosphorylated in response to PKA activation. This work demonstrates PKA-dependent phosphorylation of Na^+,K^+-ATPase in native cells, which are a major physiological target of the cAMP-PKA signaling pathway. Previous studies demonstrated that phosphorylation of the Na^+,K^+-ATPase α subunit can be catalyzed by PKA itself because (*a*) the Na^+,K^+-ATPase α subunit displays a single PKA consensus site, which is phosphorylated by PKA on purified enzyme preparation [7, 10, 11], and (*b*) the removal of this PKA site abolishes PKA phosphorylation of the α subunit in transfected COS-7 cells [10, 11]. Although these data demonstrated that PKA activation is required for cAMP-induced phosphorylation of Na^+,K^+-ATPase in MTALs, because it is abolished by a PKA inhibitor (Fig. 3), they do not indicate whether Na^+,K^+-ATPase is directly phosphorylated by PKA. For example, increased phosphorylation might be accounted for by a PKA-dependent inhibition of protein phosphatase(s) [33].

Protein kinase A-mediated phosphorylation of Na^+,K^+-ATPase α subunit occurred equally well under hypoxic and under well-oxygenated conditions, whereas in the former condition, Na^+,K^+-ATPase activity was inhibited and in the later condition it was stimulated. An inhibition of Na^+,K^+-ATPase activity in response to the phosphorylation of its α subunit by PKA has been reported [6, 10]. In one report, the inhibitory effect of PKA observed *in vitro* was related to the presence of the detergent required for PKA phosphorylation of the purified enzyme, which, in turn, inactivates Na^+,K^+-ATPase [6]. In the other report, PKA phosphorylation-dependent inhibition of the hydrolytic activity of Na^+,K^+-ATPase was observed in transfected COS-7 cells [10]. However, these results show that PKA phosphorylation of the Na,K-ATPase is not sufficient *per se* to inhibit Na^+,K^+-ATPase activity in intact cells. Indeed, in hypoxic MTALs, mepacrine abolished the inhibitory effect of cAMP on Na^+,K^+-ATPase activity but did not alter cAMP-induced phosphorylation of Na^+,K^+-ATPase (Fig. 5). This finding suggests that the inhibitory mechanism triggered by PLA_2 stimulation applies to Na^+,K^+-ATPase units that had been phosphorylated beforehand through PKA stimulation.

Although these data do not provide a clear-cut demonstration, they suggest that phosphorylation of the Na^+,K^+-ATPase α subunit might be linked to its stimulation observed under oxygenated conditions. (*a*) Stimulation of Rb^+ uptake and phosphorylation of Na^+,K^+-ATPase occurred within the same range of cAMP concentrations (Fig. 8). (*b*) They also occurred with the same time course (Fig. 7). (*c*) Both processes were curtailed by H89 (Fig. 6), and (*d*) stimulation of Rb^+ uptake was linearly related and cosaturated with the level of phosphorylation Na^+,K^+-ATPase α subunit (Fig. 9). It is likely that PKA phosphorylation does not directly stimulate Na^+,K^+-ATPase activity, but rather, acts as a permissive post-translational modification, allowing a stimulatory effect of putative cell-specific cofactor(s). This interpretation is supported by the following observations: (*a*) the activity of Na^+,K^+-ATPase purified from shark rectal gland is increased in response to its phosphorylation by PKA *in vitro*, whereas the activity of Na^+,K^+-ATPase purified from pig kidney remains unchanged [9]; and (*b*) the effect of PKA activators on the Na^+/H^+ exchanger requires the presence of associated regulatory proteins [34].

In conclusion, under well-oxygenated conditions, PKA activation increases the phosphorylation level and the activity of Na^+,K^+-ATPase, which likely participates to increase sodium reabsorption by MTALs. When oxygen availability is restricted, this stimulatory pathway is overridden by the activation of a PLA_2-mediated pathway leading to an inhibition of Na^+,K^+-ATPase activity. This latter mechanism might be important to insure cell survival under pathological conditions.

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APPENDIX

Abbreviations used in this article are: BSA, bovine serum albumin; db-cAMP, dibutyryl-adenosine 3′,5′-cyclic phosphate; 8br-cAMP, 8-bromo-cAMP; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; PAGE, polyacrylamide gel electrophoresis; MTAL, medullary thick ascending limb; PKA, protein kinase A; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; Tris, tris(hydroxymethyl) aminomethane.

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